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ORIGINAL ARTICLE

Genetic diversity, reassortment, and recombination of mammalian orthoreoviruses from Japanese porcine fecal samples

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Abstract

Mammalian orthoreoviruses (MRVs) are non-enveloped double-stranded RNA viruses with a broad host range. MRVs are prevalent worldwide, and in Japan, they have been isolated from various hosts, including humans, dogs, cats, wild boars, and pigs, and they have also been found in sewage. However, Japanese porcine MRVs have not been genetically characterized. While investigating porcine enteric viruses including MRV, five MRVs were isolated from the feces of Japanese pigs using MA104 cell culture. Genetic analysis of the *S1* gene revealed that the Japanese porcine MRV isolates could be classified as MRV-2 and MRV-3. Whole genome analysis showed that Japanese porcine MRVs exhibited genetic diversity, although they shared sequence similarity with porcine MRV sequences in the DDBJ/EMBL/GenBank database. Several potential intragenetic reassortment events were detected among MRV strains from pigs, sewage, and humans in Japan, suggesting zoonotic transmission. Furthermore, homologous recombination events were identified in the *M1* and *S1* genes of Japanese porcine MRV. These findings imply that different strains of Japanese porcine MRV share a porcine MRV genomic backbone and have evolved through intragenetic reassortment and homologous recombination events.

Introduction

Mammalian orthoreovirus (MRV) is a species of the genus *Orthoreovirus* within the family *Reoviridae*, which also includes the species *Avian orthoreovirus*, *Baboon orthoreovirus*, *Broome orthoreovirus*, *Mahlapitsi orthoreovirus*, *Nelson Bay orthoreovirus*, *Neoavian orthoreovirus*, *Piscine orthoreovirus*, *Reptilian orthoreovirus*, and *Testudine orthoreovirus* (https://talk.ictvonline.org/ictv-reports/ictv_online_report/dsrna-viruses/w/reoviridae/1672/family-spinareoviridae as of July 2022). Orthoreoviruses are non-enveloped viruses with icosahedral symmetry. The virions have a diameter of 60–80 nm and contain 10 double-stranded (ds) RNA genome segments containing three large (*L1*, *L2*, and *L3*), three medium (*M1*, *M2*, and *M3*), and four small (*S1*, *S2*, *S3*, and *S4*) genes that encode lambda (λ), mu (μ), and sigma (σ) proteins, respectively [1]. The outer capsid σ1 protein encoded by the *S1* gene is a serotype-specific antigen of orthoreoviruses that is recognized by neutralizing antibodies [1]. Currently, four MRV serotypes – types 1 to 4 – whose prototype strains are Lang (T1L), Jones (T2J), Dearing (T3D), Abney (T3A), and Ndelle (T4N), have been recognized based on

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the antigenic reactivity of the σ1 protein and genetic relatedness of the *S1* gene [1, 2].

MRVs are prevalent worldwide and infect various mammals, including humans and pigs [1]. MRV infection is thought to be common and usually asymptomatic in humans; however, there have been reports of sporadic MRV cases with respiratory or gastrointestinal disorders [3, 4]. Recently, more-severe symptoms, including neurological and acute respiratory diseases, have been reported [5–10]. Similarly, porcine MRV infections are asymptomatic in most cases, but severe outbreaks of diarrhea in pigs, caused by MRV alone or by coinfection with other pathogens, have been reported in China, South Korea, the United States, and Italy [11–16].

Reassortant MRVs are easily generated during coinfection of the same host, even between different serotypes, owing to their segmented genomes [17]. Genetic reassortants contribute to sequence diversity and genetic evolution, resulting in increased virulence and expansion of the host range of the virus [17].

In Japan, there have been two studies on the isolation of MRV type 1 (MRV-1) and MRV type 2 (MRV-2) from the respiratory tracts of pigs with respiratory disease and fecal specimens of pigs with and without diarrhea, respectively [18, 19]. However, those reports do not contain any genetic information on the isolates, and sequence data for porcine MRV in Japan are not available. Recently, we isolated MRV type 3 (MRV-3) from a wild boar in Japan and performed genetic analysis [20]. In addition, while investigating swine enteric viruses, we isolated five MRVs from pig fecal samples, using cell culture. Here, to explore the diversity of Japanese porcine MRVs, we characterized MRVs from feces of Japanese pigs.

Materials and methods

Collection of fecal samples from pigs

Fecal samples from 230 domestic pigs (30 to 70 days old) with ($n = 10$) and without ($n = 220$) diarrhea on 13 farrow-to-finish operation farms were collected between 2017 and 2021 for enteric virus research in Miyazaki prefecture (Kyusyu Island), Tottori prefecture (western region of the main island), and Kanagawa and Ishikawa prefectures (central region of the main island). Single samples from individual animals were used throughout the study. Samples were diluted 1:9 (w/v) with Eagle's minimal essential medium (EMEM) (Nissui, Tokyo, Japan) and centrifuged at 3,000 × *g* for 10 min. Supernatants were stored at -80 °C and subsequently used for virus isolation and genome analysis.

Virus isolation

The supernatant was activated by adding 20 µg of trypsin (Sigma-Aldrich, catalog no. 0303; MO, USA) per mL and incubating for 1 h at 37 °C. The samples were inoculated onto the monkey kidney epithelial cell line MA-104 for virus isolation. Confluent monolayers of MA-104 cells in 24-well plates were washed twice with EMEM and inoculated with 0.1 mL of supernatant of the activated fecal sample. After adsorption for 60 min at 37 °C, the cells were washed three times with EMEM containing 0.8 µg of trypsin per mL and incubated for 7 days at 37 °C and 5% CO_2. If no cytopathic effect (CPE) was observed after 7 days of incubation, the cells and supernatant were frozen and thawed three times and harvested, and the subsequent passages were carried out in the same manner.

Identification of MRVs using reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from the supernatants of cell cultures showing CPE using TRIzol LS Reagent (Life Technologies, Carlsbad, CA, USA). Reverse transcription was performed using PrimeScript™ Reverse Transcriptase (TaKaRa Bio, Otsu, Japan) with random primers. PCR was performed using primers targeting a portion of the *L1* gene of MRV [21]. The RT-PCR products were resolved by electrophoresis on a 2% agarose gel.

Construction of cDNA libraries and deep sequencing

Viral RNA extracted from cell culture supernatants that were positive by MRV RT-PCR was treated with DNase I (Takara Bio). Subsequently, cDNA libraries were constructed for deep sequencing using an NEBNext Ultra II RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. After assessing the library quantity on a Qubit® 4.0 Fluorimeter (Invitrogen, Carlsbad, CA, USA), deep sequencing was performed using a MiSeq benchtop sequencer (Illumina, San Diego, CA, USA) with paired-end reads of 151 nucleotides. Sequence analysis was performed using MiSeq Reporter v2.5 (Illumina) to generate FASTQ-formatted sequence data, which were imported into CLC Genomics Workbench 7.5.5 (CLC bio, Aarhus, Denmark). The sequence data were trimmed, and low-quality sequences were removed. Subsequently, the processed sequence data were assembled into contigs using the *de novo* assembly command in CLC Genomics Workbench.

Genome analysis

The Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search and compare MRV genome segments. The complete genome sequences of the 10 MRV segments were aligned with MRV sequences in the GenBank/EMBL/DDBJ database, using ClustalW [22]. Pairwise sequence identity calculations were performed for each genome segment using CLC Genomics Workbench. Phylogenetic analysis was performed on nucleotide sequences of all segments using the maximum-likelihood method with the best-fit model (the GTR+G+I model for the *L1, L2, L3, M1, M2, M3, S2*, and *S3* phylogenetic trees and the GTR+G model for the *S1* and *S4* phylogenetic trees) in MEGA7 [23]. The reliability of the phylogenetic trees was evaluated by performing 1000 replicates of bootstrap analysis [24]. Recombination and similarity plot analyses were performed using the Recombination Detection Program (RDP) and SimPlot software v. 3.5.1, respectively [25, 26].

Results

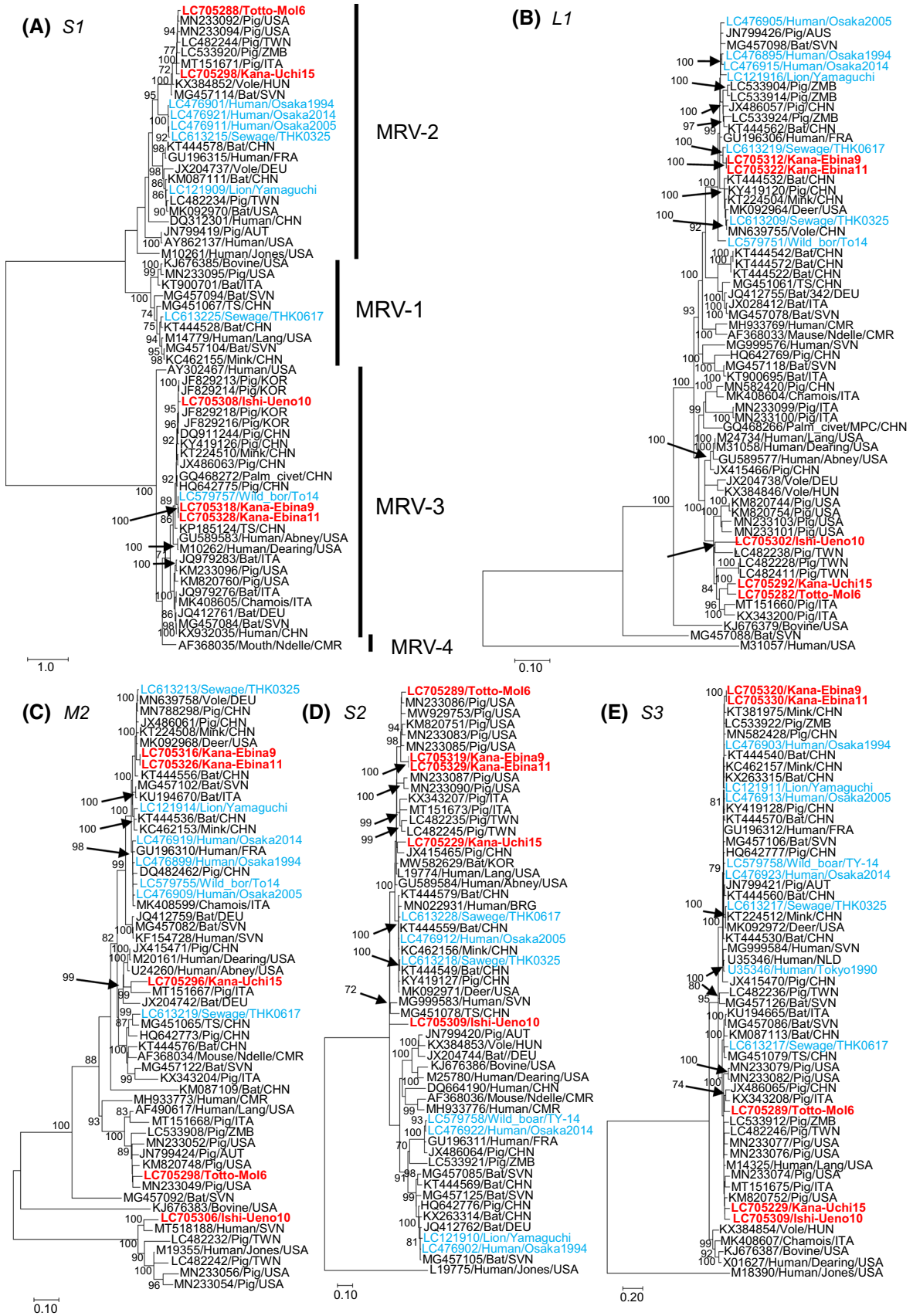
Isolation and identification of MRVs from porcine fecal samples

MA-104 cells inoculated with several of the activated fecal samples exhibited CPE after two to three days (when the cells were in the second or third passages). To detect the MRV genome in the supernatants of cell cultures showing CPE, RT-PCR was performed using a primer pair targeting the *L1* gene. Amplicons of the expected size were found in five samples from pigs without diarrhea. These five samples were subjected to deep sequencing, and the nearly complete genome sequence of 10 segments was determined. The nucleotide sequences of the five Japanese MRV strains obtained in this study (Totto-MoI6 from a healthy 59-day-old pig from Tottori prefecture in 2018, Kana-Uchi-15 from a healthy 70-day-old pig from Kanagawa prefecture in 2020, Ishi-Ueno-10 from a healthy 39-day-old pig from Ishikawa prefecture in 2021, and Kana-Ebina-9 and Kana-Ebina-11 from two healthy 70-day-old pigs from the same farm in Kanagawa Prefecture in 2021) were deposited in the DDBJ/EMBL/GenBank database under the accession numbers LC705282–LC705331.

Phylogenetic analysis and pairwise nucleotide comparison

Phylogenetic analysis of the *S1* genes of Japanese porcine MRVs and MRVs obtained from the DDBJ/EMBL/GenBank database revealed that Tottori-MoI6 and Kana-Uchi-15 clustered with porcine MRV-2 strains, while Ishi-Ueno-10, Kana-Ebina-9, and Kana-Ebina-11 branched with MRV-3 viruses from pigs, minks, bats, masked palm civets, wild boars, common tree shrews, and humans (Fig. 1A, Supplementary Fig. S1G). To confirm the sequence relationship to Japanese porcine MRVs, BLAST analysis was performed using *S1* gene sequences. The *S1* sequences of Totto-MoI6, Kana-Uchi-15, and Ishi-Ueno-10 exhibited sequence similarity to those of porcine MRV-2 and MRV-3 strains from the USA and South Korea, respectively. However, the *S1* gene of Kana-Uchi-15 diverged from those of other strains (≥89.1% and ≥88.3% nucleotide and amino acid sequence identity, respectively; Table 1). Notably, the *S1* genes of Kana-Ebina-9 and Kana-Ebina-11 had high sequence similarity (98.4% and 98.7% nucleotide and amino acid sequence identity, respectively) to the MRV-3 strain WB/To14, which was isolated from Japanese wild boar in Toyama prefecture, in the central region of the main island, in 2018 [20]. Phylogenetic analysis of the *L1* gene showed that Totto-MoI6, Kana-Uchi-15, and Ishi-Ueno-10 branched with porcine MRVs from the USA, Taiwan, and Italy (Fig. 1B, Supplementary Fig. S1A), respectively, and Ishi-Ueno-10 shared the highest pairwise identity with the prototype human MRV strain Lang, in addition to porcine MRV strains (Table 1). Kana-Ebina-9 and -11 branched separately from other Japanese porcine MRV strains and were closely associated with MRV strain THK0617 from Japanese sewage, with high nucleotide and amino acid sequence identity values (98.3% and 99.7%, respectively) (Fig. 1B, Supplementary Fig. S1A, Table 1).

In the M2 tree, Japanese MRVs were distantly related. Totto-MoI6 and Kana-Uchi-15 formed a cluster with porcine MRVs from the USA, Zambia, and Italy. Ishi-Ueno-10 branched with a human MRV from Slovenia and was distantly related to other Japanese MRV strains, including those isolated from humans, wild boars, lions, and sewage (Fig. 1C, Supplementary Fig. S1E). The nucleotide sequence identity between Ishi-Ueno-10 and other Japanese MRV strains was ≤76.7%, while the amino acid sequence identity was 94.1–96.5% (Table 2). Kana-Ebina-9 and -11 formed a cluster with porcine, vole, mink, deer, human (Japanese), and sewage MRV strains (Fig. 1C, Supplementary Fig. S1E). The S2 tree also showed diversity among the Japanese MRVs (Fig. 1D, Supplementary Fig. S1E). Totto-MoI-6 and Kana-Ebina-9 and -11, and Kana-Uchi-15 branched with the American and Chinese porcine MRVs, respectively. Ishi-Ueno-10 diverged from other MRV strains, exhibiting ≤89.6% identity in their nucleotide sequences. However, Ishi-Ueno shared high amino acid sequence similarity (97.6–97.9%) with mink, tree shrew, and human MRV strains (Fig. 1D, Supplementary Fig. S1H, Table 1). Regarding *S3* gene analysis, Totto-MoI6, Kana-Uchi-15, and Ishi-Ueno-10 were related to porcine MRV strains and the


(A) S1
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MN233094/Pig/USA
LC482244/Pig/TWN
LC533920/Pig/ZMB
MT151671/Pig/ITA
LC705298/Kana-Uchi15
KX384852/Vole/HUN
MG457114/Bat/SVN
LC476901/Human/Osaka1994
LC476921/Human/Osaka2014
LC476911/Human/Osaka2005
LC613215/Sewage/THK0325
KT444578/Bat/CHN
GU196315/Human/FRA
JX204737/Vole/DEU
KM087111/Bat/CHN
LC121909/Lion/Yamaguchi
LC482234/Pig/TWN
MK092970/Bat/USA
DQ312301/Human/CHN
JN799419/Pig/AUT
AY862137/Human/USA
M10261/Human/Jones/USA
KJ676385/Bovine/USA
MN233095/Pig/USA
KT900701/Bat/ITA
MG457094/Bat/SVN
MG451067/TS/CHN
LC613225/Sewage/THK0617
KT444528/Bat/CHN
M14779/Human/Lang/USA
MG457104/Bat/SVN
KC462155/Mink/CHN
AY302467/Human/USA
JF829213/Pig/KOR
JF829214/Pig/KOR
LC705308/Ishi-Ueno10
JF829218/Pig/KOR
JF829216/Pig/KOR
DQ911244/Pig/CHN
KY419126/Pig/CHN
KT224510/Mink/CHN
JX486063/Pig/CHN
GQ468272/Palm_civet/CHN
HQ642775/Pig/CHN
LC579757/Wild_bor/To14
LC705318/Kana-Ebina9
LC705328/Kana-Ebina11
KP185124/TS/CHN
GU589583/Human/Abney/USA
M10262/Human/Dearing/USA
JQ979283/Bat/ITA
KM233096/Pig/USA
KM820760/Pig/USA
JQ979276/Bat/ITA
MK408605/Chamois/ITA
JQ412761/Bat/DEU
MG457084/Bat/SVN
KX932035/Human/CHN
AF368035/Mouth/Ndelle/CMR
MRV-2
MRV-1
MRV-3
MRV-4
1.0
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MG457098/Bat/SVN
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LC476915/Human/Osaka2014
LC121916/Lion/Yamaguchi
LC533904/Pig/ZMB
LC533914/Pig/ZMB
JX486057/Pig/CHN
LC533924/Pig/ZMB
KT444562/Bat/CHN
GU196306/Human/FRA
LC613219/Sewage/THK0617
LC705312/Kana-Ebina9
LC705322/Kana-Ebina11
KT444532/Bat/CHN
KY419120/Pig/CHN
KT224504/Mink/CHN
MK092964/Deer/USA
LC613209/Sewage/THK0325
MN639755/Vole/CHN
LC579751/Wild_bor/To14
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KT444522/Bat/CHN
MG451061/TS/CHN
JQ412755/Bat/342/DEU
JX028412/Bat/ITA
MG457078/Bat/SVN
MH933769/Human/CMR
AF368033/Mause/Ndelle/CMR
MG999576/Human/SVN
HQ642769/Pig/CHN
MG457118/Bat/SVN
KT900695/Bat/ITA
MN582420/Pig/CHN
MK408604/Chamois/ITA
MN233099/Pig/ITA
MN233100/Pig/ITA
GQ468266/Palm_civet/MPC/CHN
M24734/Human/Lang/USA
M31058/Human/Dearing/USA
GU589577/Human/Abney/USA
JX415466/Pig/CHN
JX204738/Vole/DEU
KX384846/Vole/HUN
KM820744/Pig/USA
KM820754/Pig/USA
MN233103/Pig/USA
MN233101/Pig/USA
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LC482238/Pig/TWN
LC482228/Pig/TWN
LC482411/Pig/TWN
LC705292/Kana-Uchi15
LC705282/Totto-Mol6
MT151660/Pig/ITA
KX343200/Pig/ITA
KJ676379/Bovine/USA
MG457088/Bat/SVN
M31057/Human/USA
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(C) M2
LC613213/Sewage/THK0325
MN639758/Vole/DEU
MN788298/Pig/CHN
JX486061/Pig/CHN
KT224508/Mink/CHN
MK092968/Deer/USA
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LC705326/Kana-Ebina11
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KU194670/Bat/ITA
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KT444536/Bat/CHN
KC462153/Mink/CHN
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GU196310/Human/FRA
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KF154728/Human/SVN
JX415471/Pig/CHN
M20161/Human/Dearing/USA
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LC705296/Kana-Uchi15
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LC613219/Sewage/THK0617
MG451065/TS/CHN
HQ642773/Pig/CHN
KT444576/Bat/CHN
AF368034/Mouse/Ndelle/CMR
MG457122/Bat/SVN
KX343204/Pig/ITA
KM087109/Bat/CHN
MH933773/Human/CMR
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KX384854/Vole/HUN
MK408607/Chamois/ITA
KJ676387/Bovine/USA
X01627/Human/Dearing/USA
M18390/Human/Jones/USA
0.20

◂**Fig. 1** Phylogenetic analysis based on nearly complete *S1* (A), *L1* (B), *M2* (C), *S2* (D), and *S3* (E) gene nucleotide sequences of Japanese porcine MRVs and MRV strains obtained from the DDBJ/EMBL/GenBank databases. The phylogenetic trees were constructed using the maximum-likelihood method in MEGA7 with best-fit models (the GTR+G model for the *S1* phylogenetic tree and the GTR+G+I model for the *L1*, *M2*, *S2*, and *S3* phylogenetic trees). Bootstrap values above 70 (1,000 replicates) are shown. The bars represent the corrected genetic distances. Japanese porcine MRVs and Japanese MRVs from humans, wild boar, lion, and sewage are indicated in red and blue, respectively.

prototype MRV strain Lang, while Kana-Ebina-9 and -11 shared high similarity with the Japanese human MRV strains Osaka 1994 and Osaka 2005 and mink MRV strains from China (Fig. 1E, Supplementary Fig. S1I, Table 1). Phylogenetic analysis and pairwise comparisons of *L2*, *L3*, *M1*, *M3*, and *S4* revealed that Japanese porcine MRVs formed a cluster together with porcine MRVs from the USA, Italy, Taiwan, Zambia, and China and the human prototype Lang strain and exhibited sequence similarity to porcine strains and the prototype human strain (Supplementary Fig. S1B, C, D, F, J, Table 1). Regarding the *S4* gene, Japanese porcine MRVs were distantly related to the Japanese MRV strains from humans, wild boars, lions, and sewage, with ≥77.6% and ≥87.7% nucleotide and amino acid sequence identity, respectively (Supplementary Fig. S1J, Table 2). Pairwise comparisons of each gene among the Japanese MRVs indicated that the sequence identity values were all ≤ 92.4%, except for the *L1* genes of Totto-MoI-6 and Kana-Uchi-15 (95.3%) and all of the genes of Kana-Ebina-9 and -11 (99.3–100%) (Table 2).

Recombination analysis

Several studies have reported recombination events in porcine MRVs and avian orthoreoviruses [14, 27–29]. We therefore searched the Japanese porcine MRV sequences and sequences obtained from the DDBJ/EMBL/GenBank database for recombination events, using RDP and SimPlot software. In the sequence data obtained from the DDBJ/EMBL/GenBank database, there were four previously unreported putative recombination events strongly supported by a *P*-value cutoff of $<10^{-8}$ for the RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, and 3Seq tests in the RDP program: in *L3* (between bat and deer MRV strains and between pig MRV strains) and in *M2* (between bat MRV strains and between pig, bat, and human MRV strains) (Supplementary Fig. S2). Among the Japanese porcine MRVs, the Japanese wild boar strain WB/To14 had crossover points involving Totto-MoI-6 and Ishi-Ueno-10 in the *M1* gene (Fig. 2A). This recombination event was supported by the RDP, BootScan, MaxChi, Chimaera, and SiScan tests in the RDP program, with *P*-value cutoffs of 9.272×10^{-01}, 6.725×10^{-03}, 1.606×10^{-02}, 3.433×10^{-02}, and 5.301×10^{-03}, respectively; however, the GENECONV and 3Seq programs did not support this event. The RDP program also detected possible recombination breakpoints in the *S1* gene involving Kana-Uchi-15 and MRV strains from bats from Slovenia and humans from Japan, supported by the RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, and 3Seq tests in the RDP program, with *P*-value cutoffs of 6.342×10^{-04}, 7.730×10^{-04}, 1.375×10^{-03}, 1.616×10^{-04}, 6.431×10^{-03}, 5.440×10^{-27}, and 5.215×10^{-01} (Fig. 2B).

Discussion

In this study, while searching for porcine enteric viruses in Japan, we isolated five MRVs from five fecal samples of pigs without diarrhea by culturing them in MA-104 cells. Several other cell lines, including African green monkey kidney (Vero) cells [13–15, 19, 30–33], Madin-Darby canine kidney cells [16], primary porcine kidney cells [18], Madin-Darby bovine kidney cells [29], swine testicular cells [34], and N1380 cells [35], have been used to isolate MRVs, which suggests that MRVs can be propagated using multiple cells. Although MA-104 cells were originally used for isolating rotaviruses, they have also been used to isolate camelid MRV [36]. Thus, MA-104 cells are useful for isolating both rotaviruses and MRVs.

To obtain conclusive data for epidemiological analysis and tracing evolutionary patterns, whole-genome analysis including all of the segments is essential [37, 38]. Therefore, we analyzed all 10 gene segments of Japanese porcine MRVs together with those of MRVs with sequences in the DDBJ/EMBL/GenBank database. Most of the genes of the MRVs detected in this study showed less than 97% sequence identity to those of the three most similar strains present in the databases, demonstrating that the MRVs described in this paper are different from all the other strains found until now. Phylogenetic analysis using nearly complete nucleotide sequences of the *S1* gene showed that Japanese porcine MRVs clustered with the MRV-2 and MRV-3 strains. MRV-1 and MRV-2 have been reported previously in Japanese pigs [18, 19]. However, MRV-3 has only been isolated from wild boars and dogs [26, 39]. Thus, this is the first report of the identification of MRV-3 in Japanese pigs. The MRV-2 strains Totto-MoI6 and Kana-Uchi-15 shared only 91.7% nucleotide and 90.9% amino acid and 89.1% nucleotide and 88.3% amino acid sequence identity, respectively, with the most similar strains from the DDBJ database. Therefore, we could not determine the origin of the *S1* gene of these strains. Furthermore, their *S1* genes shared a low sequence similarity (85.8% nucleotide and 83.3% amino acid identity) with Japanese human, lion, and sewage MRV strains and low nucleotide and amino acid similarity (65.4–69.4% and

Table 1 Nucleotide sequence identity values, calculated using the basic local alignment search tool (BLAST), from comparisons of Japanese porcine mammalian orthoreoviruses to the three most similar

Segment	Totto-Mol-6/2018				Kana-Uchi-15/2020			
	Strain (nucleotide)	%	Strain (amino acid)	%	Strain (nucleotide)	%	Strain (amino acid)	%
L1	KX343200/Pig/224660-4/ITA/2015/T3	92.4	MT151660/Pig/90178-3/ITA/2018/T2	98.6	MT151660/Pig/90178-3/ITA/2018/T2	92.9	MT151660/Pig/90178-3/ITA/2018/T2	98.7
	MT151660/Pig/90178-3/ITA/2018/T2	92.4	MN233103/Pig/4476/USA/2014/T3	98.3	KX343200/Pig/224660-4/ITA/2015/T3	92.7	MN233103/Pig/4476/USA/2014/T3	98.7
	LC482228/Pig/sR1521/TWN/2015/T2	91.6	KM820754/Pig/FS-03/USA/2014/T3	98.3	LC482238/Pig/sR1590/TWN/2015/T2	91.9	KM820754/Pig/FS-03/USA/2014/T3	98.7
L2	AF378003/Human/Lang/USA/1953/T1	94.8	AF378003/Human/Lang/USA/1953/T1	98.4	AF378003/Human/Lang/USA/1953/T1	93.6	AF378003/Human/Lang/USA/1953/T1	97.8
	MW929747/Pig/USA/2018/T1	91.9	AF378008/Bovine/Maryland/USA/1961/T3	98.3	MW929747/Pig/USA/2018/T1	90.9	AF378008/Bovine/Maryland/USA/1961/T3	97.7
	MN233038/Pig/32755/USA/2014/T2	91.5	AF378007/Murine/FRA/1961/T3	97.4	MN233036/Pig/66848/USA/2005/T2	90.7	AF378007/Murine/FRA/1961/T3	96.9
L3	KX343202/Pig/224660-4/ITA/2015/T3	93.3	JX415472/Pig/SHR-A/CHN/2011/T1	98.0	AF129820/Human/Lang/USA/1953/T1	93.8	JX415472/Pig/SHR-A/CHN/2011/T1	98.8
	AF129820/Human/Lang/USA/1953/T1	93.0	AF129820/Human/Lang/USA/1953/T1	98.0	JX415472/Pig/SHR-A/CHN/2011/T1	93.5	GU589579/Human/Abney/USA/1955/T3	98.8
	GU589579/Human/Abney/USA/1955/T3	92.5	GU589579/Human/Abney/USA/1955/T3	98.0	GU589579/Human/Abney/USA/1955/T3	93.3	AF129820/Human/Lang/USA/1953/T1	98.6
M1	AF461682/Human/Lang/USA/1953/T1	92.7	MG457101/Bat/S1-MRV04/SIN/2009/T1	96.3	MN233060/Pig/66848/USA/2005/T2	94.1	MN233060/Pig/66848/USA/2005/T2	96.5
	JX415468/Pig/SHR-A/CHN/2011/T1	92.0	JX415468/Pig/SHR-A/CHN/2011/T1	96.1	AF461682/Human/Lang/USA/1953/T1	93.0	AY428872/Pig/NLD/1971/T1	95.9
	AF461683/Human/Dearing/USA/1955/T3	91.6	AY428872/Pig/NLD/1971/T1	96.1	LC482241/Pig/sR1590/TWN/2015/T2	92.5	AF461682/Human/Lang/USA/1953/T1	95.9
M2	MN233050/Pig/4476-1/USA/2014/T3	94.8	MN233050/Pig/4476-1/USA/2014/T3	99.3	U24260/Human/Abney/USA/1955/T3	91.4	MT151667/Pig/90178-3/ITA/2018/T2	96.9
	KM820748/Pig/BM-100/USA/2014/T3	94.6	MN233049/Pig/4476-2/USA/2014/T3	99.3	JX415471/Pig/SHR-A/CHN/2011/T1	91.3	MG451065/Tree shrew/CHN/2011/T1	96.9
	JN799424/Pig/729/AUT/1998/T2	94.3	P11077/Human/Lang/USA/1953/T1	99.0	M20161/Human/Dearing/USA/1955/T3	91.0	KT444576/Bat/WIV3/CHN/2011/T2	96.9
M3	MN233048/Pig/32755/USA/2014/T2	93.8	MN233048/Pig/32755/USA/2014/T2	97.2	KM820749/Pig/BM-100/USA/2014/T3	92.2	MN233042/Pig/4476/USA/2014/T3	98.2
	KM820749/Pig/BM-100/USA/2014/T3	91.5	MN233042/Pig/4476/USA/2014/T3	96.0	MN233042/Pig/4476/USA/2014/T3	91.7	JX486062/Pig/GD-1/CHN/2012/T3	97.5
	MN233042/Pig/4476/USA/2014/T3	90.8	JX486062/Pig/GD-1/CHN/2012/T3	95.8	MN233043/Pig/66848/USA/2005/T2	91.5	KM820749/Pig/BM-100/USA/2014/T3	97.5
S1	MN233092/Pig/66848/USA/2005/T2	91.7	MN233092/Pig/66848/USA/2005/T2	90.9	MN233092/Pig/66848/USA/2005/T2	89.1	LC533930/Pig/117/ZAM/2018/T2	88.3
	MN233091/Pig/32755/USA/2014/T2	90.6	LC482244/Pig/sR1590/TWN/2015/T2	88.9	MN233093/Pig/4560-2/USA/2014/T2	88.8	LC533910/Pig/85/ZAM/2018/T2	87.4
	MN233093/Pig/4560-2/USA/2014/T2	90.4	MN233091/Pig/32755/USA/2014/T2	89.6	MN233094/Pig/4560-1/USA/2014/T2	88.6	MN233092/Pig/66848/USA/2005/T2	86.3
S2	MN233086/Pig/66848/USA/2005/T2	96.1	MN233086/Pig/66848/USA/2005/T2	99.0	L19774/Human/Lang/USA/1953/T1	94.8	P03525/Human/Dearing/USA/T3	98.3
	MN233084/Pig/4543-2/USA/2014/T1	95.5	MN233083/Pig/32755/USA/2014/T2	98.8	MW582629/Pig/USA/2019/T1	94.2	KT224511/Mink/SD-14/CHN/2014/T3	98.1
	MW929753/Pig/USA/2018/T1	95.1	KM820751/Pig/BM-100/USA/2014/T3	98.6	GU589584/Human/Abney/USA/1955/T3	93.5	MG451078/Tree shrew/CHN/2012/T3	97.9
S3	M14325/Human/Lang/USA/1953/T1	94.3	LC476913/Human/Osaka2005/JPN/2005/T2	98.1	KM820762/Pig/FS-03/USA/2014/T3	94.8	MN233075/Pig/4543-1/USA/2014/T1	99.2
	KM820762/Pig/FS-03/USA/2014/T3	94.1	KF013856/Mink/HB-B/CHN/2013/T1	97.8	M14325/Human/Lang/USA/1953/T1	94.4	MN233077/Pig/4476/USA/2014/T3	99.2
	MN233076/Pig/66848/USA/2005/T2	93.7	U35346/Human/Netherlands/1/1985/T1	97.8	KM820752/Pig/BM-100/USA/2014/T3	94.1	KM820752/Pig/BM-100/USA/2014/T3	98.9
	JX415473/Pig/SHR-A/CHN/2011/T1	92.8	MT151677/Pig/90178-3/ITA/2018/T2	96.4	JX415473/Pig/SHR-A/CHN/2011/T1	94.0	MN233066/Pig/4543-1/USA/2014/T1	97.5
S4	MN233065/Pig/32755/USA/2014/T2	92.8	MN233065/Pig/32755/USA/2014/T2	95.9	MN233066/Pig/4543-1/USA/2014/T1	93.9	JX415473/Pig/SHR-A/CHN/2011/T1	97.0
	MN233066/Pig/4543-1/USA/2014/T1	92.3	MN233066/Pig/4543-1/USA/2014/T1	95.6	LC533933/Pig/117/ZAM/2018/T2	93.6	MN233065/Pig/32755/USA/2014/T2	96.4

Segment	Ishi-Ueno-10/2021				Kana-Ebina-9/2021				Kana-Ebina-11/2021			
	Strain (nucleotide)	%	Strain (amino acid)	%	Strain (nucleotide)	%	Strain (amino acid)	%	Strain (nucleotide)	%	Strain (amino acid)	%
L1	M24734/Human/Lang/USA/1953/T1	91.4	M24734/Human/Lang/USA/1953/T1	98.7	LC613219/Sewage/THK0617/JPN/2020/T1	98.3	LC613219/Sewage/THK0617/JPN/2020/T1	99.7	LC613219/Sewage/THK0617/JPN/2020/T1	98.3	LC613219/Sewage/THK0617/JPN/2020/T1	99.7
	GU589577/Human/Abney/USA/1955/T3	91.4	LC482238/Pig/sR1590/TWN/T2	98.5	GU196306/Human/Tou65/FRA/2005/T2	97.6	LC476905/Human/Osaka_2005/JPN/2005/T2	99.3	GU196306/Human/Tou65/FRA/2005/T2	97.6	LC476905/Human/Osaka_2005/JPN/2005/T2	99.3
	KM820744/Pig/BM-100/USA/2014/T3	91.3	MT151660/Pig/90178-3/ITA/2018/T2	98.4	LC476895/Human/Osaka_1994/JPN/1994/T2	97.4	KT224504/Mink/SD-14/CHN/2014/T3	99.2	LC476895/Human/Osaka_1994/JPN/1994/T2	97.3	KT224504/Mink/SD-14/CHN/2014/T3	99.2
L2	AF378003/Human/Lang/USA/1953/T1	93.8	AF378003/Human/Lang/USA/1953/T1	98.0	KM820755/Pig/FS-03/USA/2014/T3	94.3	KM820745/Pig/BM-100/USA/2014/T3	98.2	KM820755/Pig/FS-03/USA/2014/T3	94.3	KM820745/Pig/BM-100/USA/2014/T3	98.3
	MW929747/Pig/USA/2018/T1	90.8	AF378008/Bovine/Maryland/USA/1961/T3	97.8	KM820745/Pig/BM-100/USA/2014/T3	94.3	MN233035/Pig/4476/USA/2014/T3	97.4	KM820745/Pig/BM-100/USA/2014/T3	94.3	MN233035/Pig/4476/USA/2014/T3	97.5
	MN233038/Pig/32755/USA/2014/T2	90.7	MN233038/Pig/32755/USA/2014/T2	97.1	AF378003/Human/Lang/USA/1953/T1	93.0	AF378003/Human/Lang/USA/1953/T1	97.4	AF378003/Human/Lang/USA/1953/T1	93.0	AF378003/Human/Lang/USA/1953/T1	97.5
L3	AF129820/Human/Lang/USA/1953/T1	93.0	JX415472/Pig/SHR-A/CHN/2011/T1	98.4	AF129820/Human/Lang/USA/1953/T1	94.3	JX415472/Pig/SHR-A/CHN/2011/T1	98.8	AF129820/Human/Lang/USA/1953/T1	94.3	JX415472/Pig/SHR-A/CHN/2011/T1	98.8
	GU589579/Human/Abney/USA/1955/T3	93.0	GU589579/Human/Abney/USA/1955/T3	98.3	GU589579/Human/Abney/USA/1955/T3	93.8	GU589579/Human/Abney/USA/1955/T3	98.8	GU589579/Human/Abney/USA/1955/T3	93.8	GU589579/Human/Abney/USA/1955/T3	98.8
	JX415472/Pig/SHR-A/CHN/2011/T1	92.7	AF129820/Human/Lang/USA/1953/T1	98.1	JX415472/Pig/SHR-A/CHN/2011/T1	93.7	AF129820/Human/Lang/USA/1953/T1	98.6	JX415472/Pig/SHR-A/CHN/2011/T1	93.7	AF129820/Human/Lang/USA/1953/T1	98.5
M1	AF461682/Human/Lang/USA/1953/T1	93.0	LC476918/Human/Osaka2014/T2	96.1	AF461682/Human/Lang/USA/1953/T1	93.1	AF461682/Human/Lang/USA/1953/T1	95.9	AF461682/Human/Lang/USA/1953/T1	93.1	AF461682/Human/Lang/USA/1953/T1	95.9
	JX415468/Pig/SHR-A/CHN/2011/T1	92.2	AF461682/Human/Lang/USA/1953/T1	96.1	JX415468/Pig/SHR-A/CHN/2011/T1	92.3	LC476918/Human/Osaka2014/T2	95.8	JX415468/Pig/SHR-A/CHN/2011/T1	92.3	LC476918/Human/Osaka2014/T2	95.8
	AF461683/Human/Dearing/USA/1955/T3	92.1	JX415468/Pig/SHR-A/CHN/2011/T1	95.9	AF461683/Human/Dearing/USA/1955/T3	92.3	JX415468/Pig/SHR-A/CHN/2011/T1	95.7	AF461683/Human/Dearing/USA/1955/T3	92.3	JX415468/Pig/SHR-A/CHN/2011/T1	95.7
M2	MT518188/Human/SI-MRV08/SVN/2011/T2	92.5	MT518188/Human/SI-MRV08/SVN/2011/T2	98.0	KY419124/Pig/ZJ/CHN/2013/T3	97.9	LC613213/Sewage/THK0325/JPN/2020/T2	99.7	MN788298/Pig/HLJY/CHN/2017/T1	98.0	LC613213/Sewage/THK0325/JPN/2020/T2	99.7
	M19355/Human/Jones/USA/1955/T2	89.5	M19355/Human/Jones/USA/1955/T2	97.9	KT224508/Mink/SD-14/CHN/2014/T3	97.9	KT224508/Mink/SD-14/CHN/2014/T3	99.6	KY419124/Pig/ZJ/CHN/2013/T3	97.9	KT224508/Mink/SD-14/CHN/2014/T3	99.6
	MN233057/Pig/4543-1/USA/2014/T2	87.3	MW929750/Pig/USA/2018/T1	96.6	JX486061/Pig/GD-1/CHN/2012/T3	97.9	MK092968/Deer/OV204/USA/2016/T2	99.4	KT224508/Mink/SD-14/CHN/2014/T3	97.9	MK092968/Deer/OV204/USA/2016/T2	99.4
M3	KM820749/Pig/BM-100/USA/2014/T3	92.2	MN233042/Pig/4476/USA/2014/T3	96.8	KM820749/Pig/BM-100/USA/2014/T3	94.8	MN233042/Pig/4476/USA/2014/T3	98.3	KM820749/Pig/BM-100/USA/2014/T3	94.8	MN233042/Pig/4476/USA/2014/T3	98.3
	MN233042/Pig/4476/USA/2014/T3	91.7	KM820749/Pig/BM-100/USA/2014/T3	96.7	MN233042/Pig/4476/USA/2014/T3	94.2	KM820749/Pig/BM-100/USA/2014/T3	98.2	MN233042/Pig/4476/USA/2014/T3	94.2	KM820749/Pig/BM-100/USA/2014/T3	98.2
	MN233043/Pig/66848/USA/2005/T2	91.5	MN233043/Pig/66848/USA/2005/T2	96.1	MN233043/Pig/66848/USA/2005/T2	94.1	MN233043/Pig/66848/USA/2005/T2	98.1	MN233043/Pig/66848/USA/2005/T2	94.1	MN233043/Pig/66848/USA/2005/T2	98.1
S1	JF829216/Pig/KPR146/KOR/2005/T3	94.4	JF829216/Pig/KPR146/KOR/2005/T3	93.2	LC579757/Wild boar/Toyama14/JPN/2018/T3	98.4	LC579757/Wild boar/Toyama14/JPN/2018/T3	98.7	LC579757/Wild boar/Toyama14/JPN/2018/T3	98.4	LC579757/Wild boar/Toyama14/JPN/2018/T3	98.7
	JF829217/Pig/KPR150/KOR/2005/T3	94.3	JF829217/Pig/KPR150/KOR/2005/T3	93.0	JX486063/Pig/GD-1/CHN/2012/T3	92.2	JX486063/Pig/GD-1/CHN/2012/T3	94.5	JX486063/Pig/GD-1/CHN/2012/T3	92.2	JX486063/Pig/GD-1/CHN/2012/T3	94.5
	JF829215/Pig/KPR183/KOR/2005/T3	94.2	JF829218/Pig/KPR155/KOR/2005/T3	92.8	KT224510/Mink/SD-14/CHN/2014/T3	92.0	KT444558/Bat/WIV7/CHN/2011/T3	94.5	KT224510/Mink/SD-14/CHN/2014/T3	92.0	KT444558/Bat/WIV7/CHN/2011/T3	94.5
S2	KT224511/Mink/SD-14/CHN/2014/T3	89.6	KT224511/Mink/SD-14/CHN/2014/T3	97.9	MN233086/Pig/66848/USA/2005/T2	93.7	KT444529/Bat/WIV2/CHN/2007/T1	97.9	MN233086/Pig/66848/USA/2005/T2	93.9	KT444529/Bat/WIV2/CHN/2007/T1	97.9
	LC476912/Human/Osaka2005/JPN/2005/T2	89.3	MG451078/Tree shrew/CHN/2012/T3	97.6	KM820751/Pig/BM-100/USA/2014/T3	93.2	MG451078/Tree shrew/CHN/2012/T3	97.9	MN233085/Pig/4476/USA/2014/T3	93.5	MG451078/Tree shrew/CHN/2012/T3	97.9
	KT444559/Bat/WIV7/CHN/2011/T3	89.3	P03525/Human/Dearing/USA/T3	97.6	KM820761/Pig/FS-03/USA/2014/T3	93.1	KT444579/Bat/WIV3/CHN/2011/T2	97.6	KM820751/Pig/BM-100/USA/2014/T3	93.3	KT444579/Bat/WIV3/CHN/2011/T2	97.6
S3	KM820762/Pig/FS-03/USA/2014/T3	94.1	MN233075/Pig/4543-1/USA/2014/T1	98.9	LC476903/Human/Osaka/JPN/1994/T2	97.0	KF013856/Mink/HB-B/CHN/2013/T1	100	LC476903/Human/Osaka/JPN/1994/T2	96.9	KF013856/Mink/HB-B/CHN/2013/T1	100
	M14325/Human/Lang/USA/1953/T1	93.7	MG451069/Tree shrew/CHN/2012/T3	98.6	LC476913/Human/Osaka2005/JPN/2005/T2	96.7	LC476913/Human/Osaka2005/JPN/2005/T2	99.7	KF013856/Mink/HB-B/CHN/2013/T1	96.7	LC476913/Human/Osaka2005/JPN/2005/T2	99.7
	KM820752/Pig/BM-100/USA/2014/T3	93.5	KM820752/Pig/BM-100/USA/2014/T3	98.6	KF013856/Mink/HB-B/CHN/2013/T1	96.7	LC476903/Human/Osaka/JPN/1994/T2	99.7	LC476913/Human/Osaka2005/JPN/2005/T2	96.6	LC476903/Human/Osaka/JPN/1994/T2	99.7
S4	MN233067/Pig/4476/USA/2014/T3	90.6	MW929755/Pig/USA/2018/T1	97.3	MW929755/Pig/USA/2018/T1	91.7	MN233073/Pig/66848/USA/2005/T2	96.2	MW929755/Pig/USA/2018/T1	91.7	MN233073/Pig/66848/USA/2005/T2	96.2
	MN233066/Pig/4543-1/USA/2014/T1	90.2	LC482237/Pig/sR1521/TWN/2015/T2	96.4	MN233073/Pig/66848/USA/2005/T2	91.7	KM820753/Pig/BM-100/USA/2014/T3	95.9	MN233073/Pig/66848/USA/2005/T2	91.7	KM820753/Pig/BM-100/USA/2014/T3	95.9
	LC482247/Pig/sR1590/TWN/2015/T2	90.1	MN233065/Pig/32755/USA/2014/T2	96.2	KM820763/Pig/FS-03/USA/2014/T3	91.0	MW929755/Pig/USA/2018/T1	95.9	KM820763/Pig/FS-03/USA/2014/T3	91.0	MW929755/Pig/USA/2018/T1	95.9

70.4–74.1% identity, respectively) to strains belonging to MRV-2 (Table 2). As the *S1* gene segment encodes the σ1 protein, which is a specific antigen [1], these sequence differences might be associated with antigenic variations among Japanese MRV-2 strains.

In almost all of the phylogenetic trees, Japanese porcine MRVs branched in the same clusters as foreign porcine MRVs and old human prototype MRVs, which suggested that Japanese porcine MRVs shared a common origin, carrying the porcine MRV backbone. The nucleotide sequence identity values between Japanese porcine MRVs and the most closely related MRV strains were higher than the amino acid sequence identity values. This can be explained by the presence of non-synonymous mutations. Some gene segments of Japanese porcine MRVs, such as *L1*, *L2*, *L3*, *M1*, and *M2*, may have descended from the old human prototype MRVs by non-synonymous substitution. Furthermore, some gene segments of Japanese porcine MRV, such as S2 of Ishi-Ueno-10, formed individual branches and exhibited low nucleotide sequence similarity to other MRVs; thus, we could not determine the origin of the gene segments. As insufficient sequence data of MRV gene segments are available in the DDBJ/EMBL/GenBank, studies for obtaining sufficient sequence data are warranted.

Whole-genome analysis demonstrated several possible reassortment events between Japanese porcine and other MRV strains. In the *M2* gene segment, Ishi-Ueno-10 branched distantly from Japanese MRVs and formed a cluster with human MRV from Slovenia, suggesting potential zoonotic transmission. The interactions between porcine and human MRV genes result in reassortment and may generate novel viruses that are pathogenic to pigs or humans. The genomes of Kana-Ebina-9 and -11 had possible reassortment of gene segments derived from Japanese MRVs. The *S1* genes of Kana-Ebina-9 and -11 were closely related to the MRV-3 WB/To14 strain isolated from Japanese wild boars. In recent years, free-living wild boars in Japan have increased in their distribution range and population size and have thus become a viral disease reservoir and a mode of transmission of pathogens, such as classical swine fever, to domestic pigs [40–43]. The *L1*, *M2*, and *S3* gene segments of Kana-Ebina-9 and -11 had high sequence similarity to the Japanese human MRV-2 Osaka and Japanese sewage MRV strains (Table 2). Japanese sewage MRV strains shared sequence similarity with MRV-2 Osaka strains, although sewage samples were collected in the Tohoku area, situated more than 500 km from the city of Osaka. This finding suggests that MRV-2 Osaka-like strains are prevalent throughout Japan [30]. Thus, Kana-Ebina-9 and -11 may have acquired the *M2*, *S2*, and *S3* gene segments from human MRVs prevalent in Japan via direct zoonotic transmission or environmental water.

Table 2 Pairwise nucleotide and amino acid sequence identity values, calculated using the Basic Local Alignment Search Tool (BLAST), from comparisons of each segment of Japanese MRVs from pigs, wild

L1 Amino acid \ Nucleotide

	Mol-6	Uchi-15	Ueno-10	Ebina-9	Ebina-11	WB/To14	P Leo	Osaka1994	Osaka2005	Osaka2014	Sc THK0325	Sc THK0617
Totto-Mol-6/2018/T2		95.3	90.4	87.9	87.9	87.5	87.2	87.9	87.8	87.7	87.4	87.8
Kana-Uchi-15/2020/T2	99.1		90.6	87.8	87.8	87.2	87.1	87.7	87.7	87.6	87.4	87.4
Ishi-Ueno-10/2021/T3	97.6	98.0		87.2	87.1	86.5	86.1	87.1	87.0	87.2	86.7	87.0
Kana-Ebina-9/2021/T3	97.4	97.8	97.7		100	95.7	96.1	97.4	97.2	97.3	96.4	98.2
Kana-Ebina-11/2021/T3	97.4	97.8	97.7	100		95.7	96.1	97.3	97.1	97.3	96.3	98.2
Wild boar_To14/2018/JPN/T3 (LC579751)	97.5	97.9	97.8	98.9	98.9		95.3	96.5	96.0	96.4	95.5	95.7
Panthera_leo/2011/JPN/T2 (LC121916)	97.2	97.6	97.6	99.0	99.0	99.0		97.2	96.9	97.0	95.7	96.2
Human/Osaka/1994/JPN/T2 (LC476895)	97.5	97.8	97.7	99.1	99.1	99.1	99.3		98.3	99.1	97.0	97.4
Human/Osaka/2005/JPN/T2 (LC476905)	97.2	97.6	97.6	99.3	99.3	98.9	99.2	99.3		98.1	96.6	97.2
Human/Osaka/2014/JPN/T2 (LC476915)	97.5	97.9	97.7	99.1	99.1	99.1	99.2	99.5	99.2		96.9	97.4
Sewage/THK0325/2020/JPN/T2 (LC613209)	97.1	97.5	97.4	98.8	98.8	98.8	99.0	99.2	99.0	99.1		96.1
Sewage/THK0617/2020/JPN/T1 (LC613219)	97.4	97.8	97.9	99.7	99.7	99.1	99.1	99.3	99.3	99.2	99.0	

L2 Amino acid \ Nucleotide

	Mol-6	Uchi-15	Ueno-10	Ebina-9	Ebina-11	WB/To14	P Leo	Osaka1994	Osaka2005	Osaka2014	Sc THK0325	Sc THK0617
Totto-Mol-6/2018/T2		91.5	91.6	90.0	90.0	84.9	84.6	84.9	85.0	84.7	84.2	84.4
Kana-Uchi-15/2020/T2	97.1		90.9	89.3	89.1	84.4	83.9	84.4	84.6	84.1	83.7	83.9
Ishi-Ueno-10/2021/T3	97.1	96.7		89.4	89.2	84.3	83.7	84.0	84.1	83.7	83.2	83.9
Kana-Ebina-9/2021/T3	96.0	95.9	96.0		99.7	84.7	84.3	84.8	84.8	84.4	83.6	84.1
Kana-Ebina-11/2021/T3	96.0	96.0	96.1	99.9		84.5	84.5	84.8	84.9	84.4	83.6	84.2
Wild boar_To14/2018/JPN/T3 (LC579752)	96.0	95.4	95.5	95.4	95.4		98.6	96.1	95.8	95.9	94.4	98.5
Panthera_leo/2011/JPN/T2 (LC121917)	96.0	95.4	95.6	95.4	95.4	99.5		96.0	95.7	96.4	94.9	98.8
Human/Osaka/1994/JPN/T2 (LC476896)	96.0	95.4	95.8	95.6	95.7	98.7	98.8		98.8	98.7	94.9	95.9
Human/Osaka/2005/JPN/T2 (LC476906)	96.0	95.5	95.7	95.7	95.8	98.8	99.0	99.5		98.4	94.5	95.6
Human/Osaka/2014/JPN/T2 (LC476916)	96.0	95.5	95.7	95.5	95.6	98.8	98.9	99.3	99.5		95.2	96.2
Sewage/THK0325/2020/JPN/T2 (LC613210)	96.0	95.7	95.7	95.6	95.7	98.9	98.9	98.7	98.8	98.8		94.8
Sewage/THK0617/2020/JPN/T1 (LC613220)	96.0	95.4	95.6	95.4	95.4	99.5	99.7	98.8	99.0	98.9	99.0	

L3 Amino acid \ Nucleotide

	Mol-6	Uchi-15	Ueno-10	Ebina-9	Ebina-11	WB/To14	P Leo	Osaka1994	Osaka2005	Osaka2014	Sc THK0325	Sc THK0617
Totto-Mol-6/2018/T2		89.4	89.0	90.0	90.0	83.4	83.4	83.7	83.2	83.4	83.2	83.3
Kana-Uchi-15/2020/T2	97.3		92.6	91.4	91.2	83.8	83.6	84.0	83.6	83.4	84.0	83.4
Ishi-Ueno-10/2021/T3	96.9	97.9		90.4	90.3	83.0	82.7	82.8	82.9	82.6	83.0	82.7
Kana-Ebina-9/2021/T3	97.3	98.1	97.8		99.8	83.5	83.3	83.5	83.4	83.3	83.1	83.2
Kana-Ebina-11/2021/T3	97.3	98.1	97.8	100		83.3	83.3	83.3	83.3	83.2	83.0	83.3
Wild boar_To14/2018/JPN/T3 (LC579753)	97.5	98.1	97.6	98.0	98.0		98.8	98.1	96.1	96.0	96.3	98.7
Panthera_leo/2011/JPN/T2 (LC121918)	97.6	98.1	97.7	98.0	98.0	99.8		98.0	96.0	95.8	96.2	98.8
Human/Osaka/1994/JPN/T2 (LC476897)	97.6	98.1	97.6	98.0	98.0	99.8	99.8		96.7	96.6	97.1	97.8
Human/Osaka/2005/JPN/T2 (LC476907)	97.3	97.9	97.3	97.8	97.8	99.5	99.6	99.6		97.7	95.9	95.9
Human/Osaka/2014/JPN/T2 (LC476917)	97.5	98.0	97.5	98.0	98.0	99.6	99.7	99.7	99.6		96.0	95.9
Sewage/THK0325/2020/JPN/T2 (LC613211)	97.6	98.2	97.7	98.1	98.1	99.6	99.7	99.7	99.5	99.6		96.2
Sewage/THK0617/2020/JPN/T1 (LC613221)	97.7	98.2	97.7	98.1	98.1	99.8	99.9	99.9	99.7	99.8	99.8	

M1 Amino acid \ Nucleotide

	Mol-6	Uchi-15	Ueno-10	Ebina-9	Ebina-11	WB/To14	P Leo	Osaka1994	Osaka2005	Osaka2014	Sc THK0325	Sc THK0617
Totto-Mol-6/2018/T2		89.0	88.2	88.2	88.2	89.8	88.7	89.4	89.5	89.9	89.3	89.5
Kana-Uchi-15/2020/T2	94.0		88.9	88.8	88.7	89.9	89.0	89.8	89.5	89.8	88.9	89.0
Ishi-Ueno-10/2021/T3	94.2	94.0		89.5	89.5	90.5	88.7	89.8	89.6	90.5	89.5	89.5
Kana-Ebina-9/2021/T3	93.5	93.9	93.6		99.9	89.9	88.6	89.5	89.4	90.0	89.5	89.3
Kana-Ebina-11/2021/T3	93.5	93.9	93.6	100		90.0	88.5	89.5	89.5	90.1	89.6	89.3
Wild boar_To14/2018/JPN/T3 (LC579754)	95.5	95.4	95.1	95.1	95.1		93.3	94.9	95.0	95.7	94.7	94.8
Panthera_leo/2011/JPN/T2 (LC121913)	94.8	94.8	95.0	94.6	94.6	97.4		96.6	96.6	95.4	93.5	94.4
Human/Osaka/1994/JPN/T2 (LC476898)	95.2	95.4	95.4	95.2	95.2	98.1	98.5		98.1	96.9	94.8	95.7
Human/Osaka/2005/JPN/T2 (LC476908)	95.5	95.8	95.7	95.4	95.4	98.1	98.8	99.2		96.8	94.7	95.6
Human/Osaka/2014/JPN/T2 (LC476918)	96.2	95.9	96.1	95.8	95.8	98.8	98.6	99.1	99.3		95.1	96.5
Sewage/THK0325/2020/JPN/T2 (LC613212)	95.4	95.1	95.2	95.0	95.0	97.8	97.4	97.8	98.1	98.8		94.2
Sewage/THK0617/2020/JPN/T1 (LC613222)	94.8	94.7	94.6	94.3	94.3	97.0	96.3	96.9	97.3	97.4	96.6	

M2 Amino acid \ Nucleotide

	Mol-6	Uchi-15	Ueno-10	Ebina-9	Ebina-11	WB/To14	P Leo	Osaka1994	Osaka2005	Osaka2014	Sc THK0325	Sc THK0617
Totto-Mol-6/2018/T2		83.2	76.7	84.2	84.4	83.6	83.9	84.1	84.2	84.0	83.3	83.8
Kana-Uchi-15/2020/T2	95.6		75.2	86.9	87.1	87.3	87.4	87.9	87.9	87.8	86.6	86.3
Ishi-Ueno-10/2021/T3	96.5	94.1		75.7	75.8	75.7	76.0	75.6	75.8	75.3	74.7	75.8
Kana-Ebina-9/2021/T3	97.6	96.1	95.6		99.8	94.7	95.1	95.8	96.0	95.6	96.9	97.8
Kana-Ebina-11/2021/T3	97.6	96.1	95.6	100		94.5	95.3	95.6	95.7	95.3	96.8	97.6
Wild boar_To14/2018/JPN/T3 (LC579754)	97.9	96.3	95.9	99.4	99.4		95.3	97.6	97.7	97.1	94.2	88.5
Panthera_leo/2011/JPN/T2 (LC121914)	97.9	96.3	96.1	99.4	99.4	99.7		95.9	95.9	95.5	94.8	88.0
Human/Osaka/1994/JPN/T2 (LC476899)	98.0	96.2	95.9	99.6	99.6	99.6	99.6		98.6	98.1	95.2	88.6
Human/Osaka/2005/JPN/T2 (LC476909)	98.0	96.5	96.1	99.6	99.6	99.9	99.9	99.7		97.9	95.4	88.8
Human/Osaka/2014/JPN/T2 (LC476919)	97.9	96.5	96.1	99.4	99.4	99.7	99.7	99.7	99.9		95.2	88.6
Sewage/THK0325/2020/JPN/T2 (LC613213)	97.9	96.3	95.9	99.7	99.7	99.7	99.7	99.6	99.9	99.7		87.8
Sewage/THK0617/2020/JPN/T1 (LC613223)	97.5	96.5	95.9	98.3	98.3	98.6	98.6	98.5	98.7	98.6	98.6	

M3 Amino acid \ Nucleotide

	Mol-6	Uchi-15	Ueno-10	Ebina-9	Ebina-11	WB/To14	P Leo	Osaka1994	Osaka2005	Osaka2014	Sc THK0325	Sc THK0617
Totto-Mol-6/2018/T2		89.1	88.6	89.0	89.0	83.0	83.1	84.0	83.6	81.7	83.1	82.0
Kana-Uchi-15/2020/T2	94.7		90.1	92.2	92.2	82.7	83.0	83.0	82.6	80.8	82.1	81.1
Ishi-Ueno-10/2021/T3	94.0	95.4		90.2	90.2	83.0	83.1	83.8	83.1	81.7	82.5	81.5
Kana-Ebina-9/2021/T3	94.6	97.1	95.6		100	83.9	83.1	84.0	84.1	81.4	83.0	82.1
Kana-Ebina-11/2021/T3	94.6	97.1	95.6	100		83.9	83.1	84.0	84.1	81.4	83.0	82.1
Wild boar_To14/2018/JPN/T3 (LC579756)	94.0	93.2	93.5	93.6	93.6		98.3	96.7	96.2	94.2	94.7	94.9
Panthera_leo/2011/JPN/T2 (LC121915)	93.6	92.8	93.3	93.3	93.3	98.8		96.5	95.6	94.0	94.7	95.0
Human/Osaka/1994/JPN/T2 (LC476900)	94.5	93.6	93.9	94.0	94.0	99.0	98.6		96.5	95.0	95.1	95.0
Human/Osaka/2005/JPN/T2 (LC476910)	94.7	93.8	93.5	94.3	94.3	98.3	97.9	99.0		93.7	94.9	94.1
Human/Osaka/2014/JPN/T2 (LC476920)	94.5	93.9	93.9	94.0	94.0	98.8	98.3	99.5	98.8		94.7	95.8
Sewage/THK0325/2020/JPN/T2 (LC613214)	94.3	93.5	93.8	93.9	93.9	98.6	98.2	99.3	98.9	99.0		95.1
Sewage/THK0617/2020/JPN/T1 (LC613224)	94.9	94.0	94.0	94.5	94.5	98.9	98.5	99.2	98.5	98.9	98.8	

S1 Amino acid \ Nucleotide

	Mol-6	Uchi-15	Ueno-10	Ebina-9	Ebina-11	WB/To14	P Leo	Osaka1994	Osaka2005	Osaka2014	Sc THK0325	Sc THK0617
Totto-Mol-6/2018/T2		85.8	39.1	39.9	40.0	39.6	65.4	68.0	67.8	68.0	66.7	52.3
Kana-Uchi-15/2020/T2	83.3		40.3	41.5	41.4	40.9	65.6	69.4	68.9	69.3	68.1	53.9
Ishi-Ueno-10/2021/T3	23.7	24.5		86.8	86.7	87.0	40.8	41.2	40.7	41.0	40.6	36.8
Kana-Ebina-9/2021/T3	24.1	24.3	88.4		99.9	98.2	41.9	42.1	41.8	41.5	41.2	37.9
Kana-Ebina-11/2021/T3	24.1	24.3	88.4	100		98.4	41.8	42.0	41.7	41.5	41.2	37.9
Wild boar_To14/2018/JPN/T3 (LC579757)	23.7	23.9	88.6	98.7	98.7		41.3	41.6	41.2	41.1	40.7	37.9
Panthera_leo/2011/JPN/T2 (LC121909)	68.1	69.4	25.8	24.5	24.5	24.1		69.9	69.8	69.1	69.1	55.1
Human/Osaka/1994/JPN/T2 (LC476901)	71.1	73.9	25.8	25.0	25.0	75.1	24.7		96.9	97.3	95.3	54.9
Human/Osaka/2005/JPN/T2 (LC476911)	71.1	73.9	25.6	24.7	24.7	75.7	24.5	98.0		96.6	97.1	54.3
Human/Osaka/2014/JPN/T2 (LC476921)	71.1	74.1	25.8	24.7	24.7	75.1	24.5	97.6	97.2		94.9	55.0
Sewage/THK0325/2020/JPN/T2 (LC613215)	70.4	73.9	25.0	24.1	24.1	75.3	23.9	96.3	97.8	95.9		54.5
Sewage/THK0617/2020/JPN/T1 (LC613225)	45.9	48.3	22.7	23.5	23.5	51.5	23.1	52.4	51.9	52.6	51.9	

S2 Amino acid \ Nucleotide

	Mol-6	Uchi-15	Ueno-10	Ebina-9	Ebina-11	WB/To14	P Leo	Osaka1994	Osaka2005	Osaka2014	Sc THK0325	Sc THK0617
Totto-Mol-6/2018/T2		90.9	86.7	92.4	92.2	84.5	84.5	85.9	92.2	84.5	90.9	91.8
Kana-Uchi-15/2020/T2	96.2		87.2	90.6	90.7	82.6	85.4	85.8	92.0	83.3	90.2	91.1
Ishi-Ueno-10/2021/T3	96.9	96.4		86.2	86.7	83.5	83.7	84.8	89.3	84.0	86.9	88.5
Kana-Ebina-9/2021/T3	97.1	96.9	95.7		99.5	82.8	83.5	84.7	90.7	83.2	89.0	90.0
Kana-Ebina-11/2021/T3	97.1	96.9	95.7	100		83.3	83.6	84.8	91.2	83.6	88.9	90.5
Wild boar_To14/2018/JPN/T3 (LC579758)	97.9	97.6	97.6	97.1	97.1		85.4	86.7	84.3	98.6	82.4	83.8
Panthera_leo/2011/JPN/T2 (LC121910)	97.4	97.9	97.1	97.4	97.4	98.8		97.4	84.6	85.2	83.5	84.1
Human/Osaka/1994/JPN/T2 (LC476902)	97.6	98.1	97.4	97.6	97.6	99.0	99.8		85.9	86.5	84.5	85.4
Human/Osaka/2005/JPN/T2 (LC476912)	98.1	97.6	97.4	97.6	97.6	99.0	98.8	99.0		84.7	96.7	98.4
Human/Osaka/2014/JPN/T2 (LC476922)	97.9	97.6	97.6	97.1	97.1	100	98.8	99.0	99.0		82.7	84.2
Sewage/THK0325/2020/JPN/T2 (LC613216)	98.1	97.6	97.4	97.6	97.6	99.0	99.0	99.0	99.5	99.0		95.9
Sewage/THK0617/2020/JPN/T1 (LC613226)	97.9	97.4	97.1	97.4	97.4	98.8	98.6	98.8	99.3	98.8	99.3	

S3 Amino acid \ Nucleotide

	Mol-6	Uchi-15	Ueno-10	Ebina-9	Ebina-11	WB/To14	P Leo	Osaka1994	Osaka2005	Osaka2014	Sc THK0325	Sc THK0617
Totto-Mol-6/2018/T2		91.0	89.2	87.5	87.4	87.8	87.9	88.3	88.2	88.5	88.0	89.6
Kana-Uchi-15/2020/T2	97.0		91.4	86.7	86.6	87.0	88.5	87.4	87.4	88.1	87.7	90.2
Ishi-Ueno-10/2021/T3	96.2	98.6		86.9	86.8	87.3	87.1	87.7	87.2	88.1	87.9	90.4
Kana-Ebina-9/2021/T3	97.8	98.1	97.3		99.9	95.3	94.9	97.0	96.7	96.5	95.1	88.5
Kana-Ebina-11/2021/T3	97.8	98.1	97.3	100		95.2	94.8	96.9	96.6	96.4	95.0	88.4
Wild boar_To14/2018/JPN/T3 (LC579759)	96.7	97.0	97.0	97.8	97.8		94.7	96.6	96.2	97.6	95.7	88.4
Panthera_leo/2011/JPN/T2 (LC121911)	97.5	97.8	97.5	99.7	99.7	98.1		96.6	96.9	95.9	94.2	89.3
Human/Osaka/1994/JPN/T2 (LC476903)	97.5	97.8	97.0	99.7	99.7	97.5	99.5		98.3	97.8	96.1	89.0
Human/Osaka/2005/JPN/T2 (LC476913)	98.1	98.4	97.5	99.7	99.7	97.5	99.5	99.5		97.5	95.7	89.2
Human/Osaka/2014/JPN/T2 (LC476923)	97.0	97.8	97.8	98.6	98.6	99.2	98.9	98.4	98.4		96.9	89.5
Sewage/THK0325/2020/JPN/T2 (LC613217)	96.2	97.0	97.0	97.8	97.8	98.4	98.1	97.5	97.5	99.2		88.6
Sewage/THK0617/2020/JPN/T1 (LC613227)	97.3	97.5	97.3	98.4	98.4	97.3	98.6	98.1	98.6	98.1	97.3	

S4 Amino acid \ Nucleotide

	Mol-6	Uchi-15	Ueno-10	Ebina-9	Ebina-11	WB/To14	P Leo	Osaka1994	Osaka2005	Osaka2014	Sc THK0325	Sc THK0617
Totto-Mol-6/2018/T2		91.8	89.0	87.1	87.1	75.9	76.7	76.3	76.4	75.7	75.8	75.7
Kana-Uchi-15/2020/T2	94.8		88.1	87.0	87.6	75.5	77.4	76.0	75.8	75.8	76.1	76.0
Ishi-Ueno-10/2021/T3	95.3	94.8		88.5	88.2	76.5	75.3	76.8	76.6	76.3	76.7	75.3
Kana-Ebina-9/2021/T3	94.0	92.9	94.8		99.3	77.4	76.8	77.5	77.4	77.4	77.6	76.8
Kana-Ebina-11/2021/T3	94.0	92.9	94.8	100		77.2	77.4	77.3	77.1	77.2	77.4	76.9
Wild boar_To14/2018/JPN/T3 (LC579760)	86.6	86.0	87.4	87.7	87.7		93.1	94.2	93.8	94.8	94.1	93.2
Panthera_leo/2011/JPN/T2 (LC121912)	86.3	85.8	87.1	87.4	87.4	99.7		93.8	93.1	97.1	97.1	97.2
Human/Osaka/1994/JPN/T2 (LC476904)	86.0	85.5	86.9	87.1	87.1	99.5	99.2		98.0	95.6	94.7	94.2
Human/Osaka/2005/JPN/T2 (LC476914)	86.6	86.0	87.4	87.7	87.7	100.0	99.7	99.5		95.1	94.1	93.7
Human/Osaka/2014/JPN/T2 (LC476924)	85.5	84.9	86.3	86.6	86.6	98.6	98.4	98.6	98.6		98.0	97.1
Sewage/THK0325/2020/JPN/T2 (LC613209)	86.3	85.8	87.1	87.4	87.4	99.2	98.9	98.6	99.2	97.8		97.4
Sewage/THK0617/2020/JPN/T1 (LC613219)	86.6	86.0	87.4	87.7	87.7	100.0	99.7	99.5	100.0	98.6	99.2	

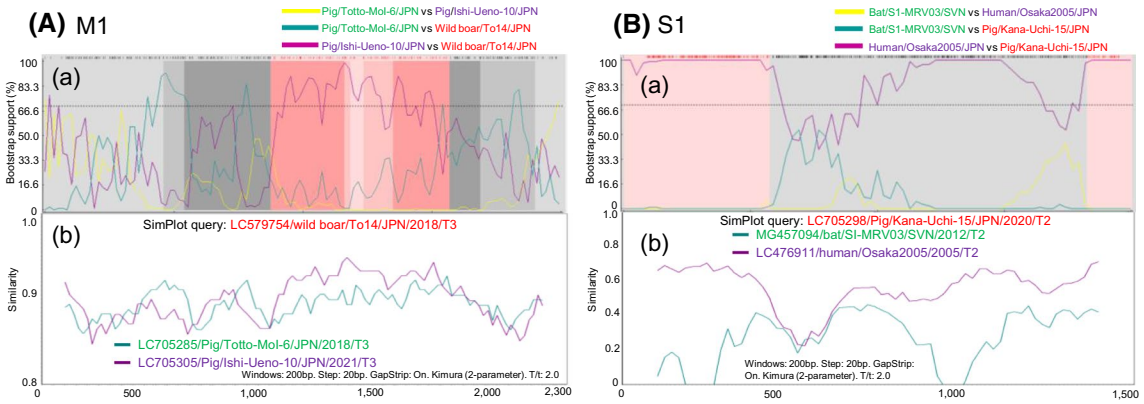

Fig. 2 (A-a) Recombination analysis of the *M1* gene segment of Pig/Totto-MoI-6/JPN vs. Pig/Ishi-Ueno-10/JPN (yellow curve), Pig/Totto-MoI-6/JPN vs. Wild boar/To14/JPN (blue-green curve), and Pig/Ishi-Ueno-10/JPN vs. Wild boar/To14/JPN (purple curve). (A-b) Similarity plots of Pig/Totto-MoI-6/JPN (blue-green curve) and Pig/Ishi-Ueno-10/JPN (purple curve), and Wild boar/To14/JPN as a query sequence, with a sliding window of 200 nucleotides and a moving step size of 20 nucleotides. (B-a) Recombination analysis of the *S1* gene segment of Bat/S1-MRV03/SVN vs. Human/Osaka2005/JPN (yellow curve), Bat/S1-MRV03/SVN vs. Pig/Kana-Uchi-15/JPN (blue-green curve), and Human/Osaka2005/JPN vs. Pig/Kana-Uchi-15/JPN (purple curve). (B-b) Similarity plots of Bat/SI-MRV03/SVN (blue-green curve) and Human/Osaka2005/2005 (purple curve), and Pig/Kana-Uchi-15/JPN as a query sequence, with a sliding window of 200 nucleotides and a moving step size of 20 nucleotides.

Regarding segmented dsRNA viruses, genetic drift and reassortment events are the primary mechanisms for the acquisition of genetic diversity; however, recombination events have also been reported in several segmented dsRNA viruses, such as rotaviruses [44–46]. Recombination events in viruses promote adaptation to a novel host species range and increase their pathogenicity [47]. Our previous study revealed a possible intra-segment recombination event in the *M2* gene of the Japanese wild boar MRV strain Wild boar/To14/JPN/2018 with a strain from a lion in a Japanese zoo and with bat strains [20]. In the present study, RDP detected crossover points in the *M1* gene of Toyama14 with Totto-MoI-6 and Ishi-Ueno-10 (Fig. 2A) and in the *S1* gene of Kana-Uchi-15 with a bat strain from Slovenia and a human strain from Japan (Fig. 2B). However, these events were not supported by the GENECONV and 3Seq programs with low *P*-value cutoffs. GENECONV and 3Seq have the least detection power among the RDP programs, but they also have the lowest false-positive rates among the RDP programs [44]. Recombination analysis using sequence data obtained from the DDBJ/EMBL/GenBank database showed that clear intragenic recombination events strongly supported by a *P*-value cutoff of $<10^{-8}$ of RDP were present in the *L3* and *M2* genes of MRVs, including porcine MRVs (Supplementary Fig. S2). These findings support the notion that intragenic recombination events might gain genomic plasticity and diversity and contribute to MRV evolution.

In conclusion, five MRV strains were isolated from fecal samples from Japanese pigs, using MA104 cells. Sequence analysis of the *S1* gene showed that the strains clustered with MRV-2 and MRV-3. Furthermore, complete genome analysis showed that Japanese porcine MRVs shared a porcine MRV genomic backbone accompanied by several possible intragenetic reassortments and homologous recombination events between MRV strains from pigs, sewage, and humans, suggesting zoonotic transmission. Overall, this study provides important information on the genetic plasticity, diversity, and evolution of porcine MRVs.

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Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Research involving human participants and/or animals This study did not involve any human participants and animals.

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